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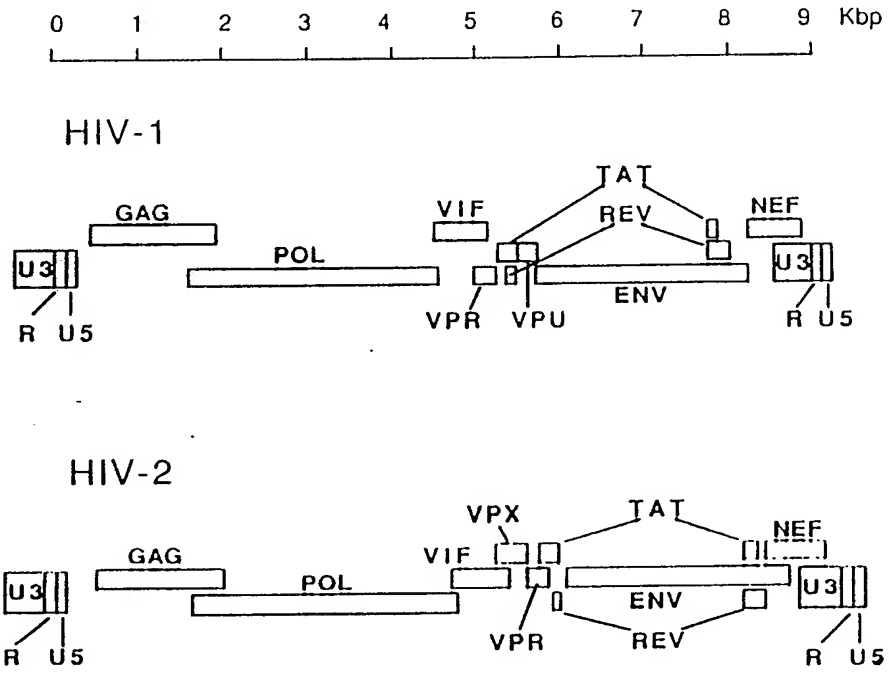

(57) Abstract

The present invention provides a mammalian stem cell transduced with one or more replicable vectors carrying antiviral DNA which by production of RNA or DNA or by expression of one or more proteins, will block replication of at least one virus infecting mammalian cells deriving from said stem cells. Also provided are methods of producing such stem cell populations and of using them to combat viral infections in mammals.

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MODIFIED MAMMALIAN STEM CELL BLOCKING VIRAL REPLICATION

This invention concerns the transduction of mammalian stem cells, particularly of the haemopoietic system, with vectors providing increased resistance to infection.

The most dangerous aspect of HIV and similar viral infections is that they can invade the cells of the immune system, particularly T and B cells, thereby rendering the infected subject at risk to opportunistic infections of all kinds in addition to the HIV infection itself. Certain viruses leading to lymphoma similarly attack the lymphocyte population and hence, again, reduce resistance to infection.

The mechanism of replication of different classes of viruses in the cell is well established. In each case, genetic material from the virus sets in train a complex sequence culminating in synthesis of new virus particles. Mammalian cells are not well equipped to combat such replication.

The present invention is based on the concept of transducing mammalian stem cells, particularly of the haemopoietic system, with replicable vectors carrying DNA which will interfere with and block viral replication. These may then be used in gene therapy as explained hereinafter.

Gene therapy has been proposed for the correction of genetic defects whereby appropriate human cells are transduced with corrected genes and transplanted into the genetically defective host. Thus, for example, Kantoff et al. (Proc Natl. Acad Sci., USA, 6563-6567,1986) have described correction of adenosine deaminase (ADA) deficiency in cultured human T and B cells by retrovirus-mediated transfer and Ferrari et al. (Nature, 15 March 1991, 1363-1366) have used human

pluripotent stem cells transduced with an ADA gene. However, it has not previously been suggested to introduce genes other than corrected endogenous genes into stem cells and, in particular, it has not been suggested to introduce genes providing an improved defence mechanism against viral infection of the transduced stem cells or their differentiated forms.

Gilboa (Retroviral Gene Transfer: Application to Human Therapy, Retroviruses and Disease, Academic Press Inc. 1989) has also suggested integrating antiviral nucleic acid into a host cell genome to combat viral infection but only in relation to differentiated host cells (in contrast to stem cells).

According to the present invention therefor there is provided a mammalian stem cell transduced with one or more replicable vectors carrying antiviral DNA which by production of RNA or DNA or by expression of one or more proteins, will block replication of at least one virus infecting mammalian cells deriving from said stem cells.

The term 'replicable vector' as used herein means a vector which by self-replication or by integration with the host genome, will replicate the DNA of the vector e.g. on cell division, and in addition will lead to production of antiviral nucleic acid or protein.

The mammalian haemopoietic stem cells differentiate into the various lymphocyte populations and thus will carry any antiviral vector into the T cell and B cell populations which are particularly at risk to infection by HIV and other viruses such as HTLV. The invention is particularly concerned with the transduction of these cells. However, stem cells of the endothelial system are also particularly suitable, for example the cells of the reticuloendothelial system in the liver which are at risk to infection by hepatitis viruses, especially the dangerous HCV type.

Methods of isolating stem cells have recently become available. They exist in very small numbers in

any mixed population. However, the expression of antigens on the cell surface during differentiation provides a means of selection using antibodies against these antigens. One such method is described in International Application WO91/09938.

Thus, the antigen CD 34 is expressed by HPC. In the first stage of differentiation into colony forming cells (e.g. CFU-GEMM) these express antigens CD33 and CD34. In the next stage of differentiation to cells of the erythroid, myelomonocytic and megakaryotic lineages, the vital BFU_E cells of the erythroid lineage carry antigens CD33 and CD 34 although these are lost in later differentiation. The myelomonocytic lineage includes CFU GM cells which carry CD33 but not CD34 although CD33 is subsequently lost. The megakaryotic lineage leads initially to CFU Mega cells which carry CD34 which is subsequently lost. Thus, monoclonal antibodies against antigen CD34 (and possibly CD33) provide one suitable method for selecting early haemopoietic cells.

A further significant system of antigens on HPC and other cells is the MHC (major histocompatibility complex) Class II group. It has been found that the majority of HPC carry an antigen termed DR and on differentiation express an antigen termed DP and then a further antigen termed DQ. Thus, the MHC Class II DR antigen is characteristic of relatively early stem cells. A novel monoclonal antibody AB-4 has been described (Cancer Research 47, 846-851, 1987) which is active against cells carrying DR antigen but not against all HPC. The DR epitopes recognized by AB-4 clearly have a more restricted expression on HPC compared with the monomorphic DR epitopes recognised by most other antibodies against monomorphic DR antigens. AB-4 is thus capable when bound to an inert support, such as magnetic particles, e.g. Dynabeads (sold by Dynal AS, Oslo, Norway), of removing from a total population of haemopoietic cells, the greater number of the more

mature cells including, in particular, B cells and any leukaemia cells while leaving a fraction of HPC in the supernatant. It has further been found that positive selection of HPC, before or after elimination of AB-4 reactive cells (negative selection), using an immobilised monoclonal antibody specific for an antigen on the stem cells, e.g. CD34 antigen, provides a method of isolation of haemopoietic stem cells. A similar negative selection may also be achieved by replacing AB-4 by a monomorphic DP or DQ specific antibody.

The preferred negative selection antibody or antibody mixture is one which is reactive with DR, DP and DQ antigens of the MHC Class II other than the monomorphic epitope of the DR antigen on HPC, especially antibodies which recognise DQ. Monoclonal antibody FN81.1 which recognises the DQ antigen is particularly suitable and 22C1 which recognises the DP antigen may also be used. These antibodies may optionally be used together with a B-cell specific antibody such as monoclonal antibody AB-1 (which recognises CD19 antigen), also described in the same publication (Cancer Research 47, 846-851, 1987) and/or an antibody against T cells such as anti-CD2 or anti-CD7 or one or more antibodies against myeloid cells such as anti-CD33, anti-CD15 or anti-CD36. Antibodies AB-4 and AB-1 are both IgM and in general IgM antibodies are preferred to IgG antibodies, partly on the basis of their ease of liberation from the cells after positive selection as described hereinafter. Both the above monoclonal antibodies were obtained from hydridomas between X63 Ag 8.653 cells and spleen cells taken from a BALB/c mouse immunised with cells taken from a patient with diffuse centroblastic B-cell lymphoma. AB-4 has been shown to recognise a monomorphic DR W52 antigen; clearly this antigen is not a monomorphic DR antigen expressed on stem cells, at least in a form capable of binding to antibody AB-4. The selected sub population of cells

obtained by positive selection with anti-CD34 magnetic beads has been successfully grown to produce blast cells. It appears that the particular mixture of naive pluripotent cells and some cells carrying the monomorphic DR antigen but not the DP and DQ antigens may be beneficial in securing blast cell growth. It is notable that pluripotent haemopoietic stem cells alone have failed to engraft in lethally irradiated mice (Jones et al, Nature, 347, 13 Sept 1990).

The positive selection antibody may, for example, be an antibody reactive with the CD34 antigen or another broadly expressed HPC antigen. More broadly active antibodies are also of value since the negative selection step will remove unwanted cells included within the wider antigen groupings and leave only the desired HPC. Thus, it is possible to use, for example, HKB1 which is a pan class II specific (Holte, H. et al. Eur.J.Immunnol. 19, 1221-1225: 1989) IgM antibody. A further candidate for positive selection is an antibody AB-3 (IgG) which also recognises a monomorphic DR antigen on stem cells (Holte, H. et al. Eur.J.Immunnol. 19, 1221-1225: 1989).

It is preferred to select stem cells at as early a stage as possible since these express very few surface antigens and are therefore serologically 'naive'. This means that they can be transplanted into hosts other than the donor without host-graft rejection.

International Application W091/09938 describes means for attaching cells to an inert support such as magnetic beads via the appropriate antibodies as discussed above and for detaching the cells subsequently by reaction with a secondary antibody or fragment thereof which by interaction with the primary antibody such as anti-CD34, causes it to detach from the cell. Thus viable populations of positively selected cells can be produced with minimal attached material which might otherwise affect viability.

Marrow replacement therapy of leukaemia is well established whereby stem cells and other pluripotent cells are selected from the total haemopoietic population and irradiation or other means are used to destroy the remaining haemopoietic cells. The stem cells are not subject to the neoplasia and may thus be reintroduced into the donor, when they rapidly locate at the correct sites for proliferation. Methods have been devised for culture of such stem cells to increase their numbers prior to transplanting (Andrews et al , Journal of Experimental Medicine, 169, 1721-1731, 1989).

Viruses generally comprise a protein envelope surrounding the genome which consists either of DNA or RNA. Many viruses possess complex structures including tails and tail fibres which aid host infection. Once inside the host cell, the virus undergoes replication which may or may not kill the cell but the outcome is proliferation of the virus particles.

Viral replication strategies are numerous. DNA viruses, that is viruses having a DNA genome, recruit the biosynthetic machinery of the host cell and substitute their genes for those of the host. In many cases, the host cell DNA is degraded by a deoxyribonuclease which is expressed by the viral genome at an early stage. The viral DNA is distinguished from the host DNA in some characteristic way, for example by hydroxymethylation of cytosine. One or more enzymes are expressed by the virus at an early stage in order to accomplish such hydroxymethylation or other characteristic functionalisation. Blocking the action of such functionalising enzymes thus provides a means of preventing replication of DNA viruses of this type since the viral DNA would be degraded together with that of the host. This can be achieved by introduction of a gene expressing an inactive form of the hydroxymethylating enzyme which will compete with that of the virus for the appropriate binding site.

In the case of RNA viruses, the host does not have appropriate biosynthetic machinery, namely an ability to synthesise nucleic acids from an RNA template. All RNA viruses must thus contain in their genome, genetic information for the synthesis of an RNA-directed RNA polymerase (an RNA synthetase) or an RNA-directed DNA polymerase (reverse transcriptase). In all cases, mRNA is eventually synthesised and directs synthesis of enzymes and RNA replication.

One strategy for blocking replication of viruses, in particular RNA viruses, is introduction into the cell of antisense oligonucleotides which are specific to regions of the viral genome which are vital to replication and/or expression. Hitherto, such oligonucleotides have been introduced by internalisation into host cells and it has never been proposed to transduce stem cells to effect manufacture within the cell.

The RNA viruses have been classified according to the relationship between their virion and mRNA. By convention, mRNA is defined as (+)RNA and its complement as (-)RNA. The four classes are as follows:

Class 1 RNA viruses (eg. poliovirus) are positive strand RNA viruses which synthesise (-)RNA to form a template for formation of (+)mRNA;

Class 2 RNA viruses (eg. rabies virus) are negative strand RNA viruses in which virion (-)RNA acts directly as the template for mRNA;

Class 3 RNA viruses (eg. reovirus) are double stranded RNA viruses in which virion (±)RNA directs asymmetric synthesis of mRNA;

Class 4 RNA viruses (eg. HIV and HTLV viruses) express genetic information in their virion (+)RNA through a DNA

intermediate which serves as a template for synthesis of mRNA.

The retroviruses such as HIV and HTLV are of particular interest and are described hereinafter in greater detail to illustrate virus life cycles and ways in which these may be blocked or disrupted in accordance with the invention.

Initially, the infecting virions bind to a receptor on the cell surface. In the case of HIV, this is the CD4 receptor and in general, HIV only invades CD4 cells such as T4 cells.

The viral (+)RNA is uncoated in the cytosol and reverse transcriptase brought in by the virus particle synthesises both the (-) and (+) strands of DNA and digests the viral (+)RNA. Thus, reverse transcriptase is essential to (a) RNA directed DNA synthesis (b) hydrolysis of viral RNA and (c) DNA directed DNA synthesis.

Thus, one means of blocking retrovirus replication is to interfere with the action of reverse transcriptase. Since this does not occur in any biosynthetic pathway of the host, there is a possibility of selective inactivation of viral replication. One possibility, which has been proposed, is to provide a reverse transcriptase inhibitor. These have normally been small molecules which can enter the cell from the body fluids. The present invention, however, uses only inhibitors which are replicated or expressed by nucleic acids.

One type of inhibitor is a nucleic acid sequence which binds to the initiation site of reverse transcriptase on the viral (+)RNA template thereby blocking transcription. Such a nucleic acid sequence should, not, however be capable of acting as a primer and should thus carry a 3'-sequence which does not hybridise.

A gene expressing a DNA binding but inactive form of reverse transcriptase may be particularly effective. The sequences of a wide range of reverse transcriptases are known and any mutation of the native gene will be effective which retains the DNA binding ability but destroys the reverse transcription activity.

In fact, initiation of transcription is effected by a transfer RNA (for example trp tRNA in Rous sarcoma virus); the 3'-OH of the tRNA acts as a primer for DNA extension of the t-RNA in the 3'-direction. Thus, one kind of inhibitor of the above type is a modified tRNA having a blocked terminal 3'-hydroxyl, eg. a non-hybridising 3'extension. Alternatively, DNA may be provided which hybridises to a position 3'- of the t-RNA binding site but is also 3'-blocked. The polymerase binding site is, in fact, near the 3'-end of the viral genomic (+)RNA. Initial 3'-extension adds a DNA sequence to the t-RNA which permits the latter to detach from the above site and hybridise to an identical sequence at the 5-end of the viral genome. A sequence immediately 3' of this sequence may thus also be blocked by an appropriately modified DNA sequence.

It will be appreciated that there are many opportunities for interference with replication of the retroviral genome. The retroviral DNA can only be transcribed when integrated into the host cell DNA. In general, integration occurs at TCAG sites in the host genome. Four to six bases at the host integration site are duplicated as in the movement of transposons. Integration may be blocked by a DNA or RNA sequence which hybridises with the sites on the viral DNA which bind to the TCAG sites.

The entire RNA genomes of HIV-1 and HIV-2 are set out, with variations, in Human Retroviruses and AIDS 1991 (Theoretical Biology and Biophysics Group T-10, Mail Stop K710, Los Alamos National Laboratory, Los Alamos, New Mexico, USA). Fig. 1 of the accompanying

drawings sets out the principal genes of the HIV-1 and HIV-2 genomes. As in all retroviruses, gene overlapping ensures the compactness of the genome. The abbreviations in Fig. 1 are explained hereinafter:

HIV Genomic Structural Elements

LTR - long terminal repeat, the DNA sequence flanking the genome of integrated proviruses; contains important regulatory regions, especially those for transcriptional start and polyadenylation.

TAR - target sequence for viral transactivation, the binding site for TAT protein and possibly cellular proteins; consists of approximately the first 45 nucleotides of the viral mRNAs in HIV-1 (or the first 100 nucleotides in HIV-2 and SIV). TAR forms a hairpin stem-loop structure with a side bulge; the bulge is necessary for TAT binding function.

RRE - (also known as CAR) REV responsible element, an RNA element encoded within the ENV region of HIV-1, consisting of approximately 200 nucleotides. The RRE is necessary for REV function; approximately seven binding sites for REV exist within the RRE RNA. Other lentiviruses (HIV-2, SIV, visna) have similar RNA element (RXRE) serving the same purpose within their LTR; RRE is the binding element for REV protein, and RXRE is the binding element for the Rex protein. RRE and RXRE thus form complex secondary structures important for specific protein binding.

CRS - cis-acting repressive sequences postulated to inhibit structural protein expression in the absence of REV. Their exact locations and function have not been defined; splice sites have been postulated as CRS sequences.

INS - "instability" RNA sequences found within the structural genes of HIV-1 and of other complex retroviruses. Multiple elements appear to exist within the genome; one of the best characterised elements spans nucleotides 414 to 631 in the GAG region of HIV-1.

Genes and Gene Products

GAG - group-specific antigens or capsid proteins; the precursor is the p55 myristoylated protein, which is processed to p17 (MAtrix) p24 (CApsid) and p7 (NucleoCapsid) proteins by the viral protease. Other small proteins are generated from the gag polyprotein.

POL - generates the viral enzymes protease, reverse transcriptase and integrase after the processing of a GAG-POL precursor polyprotein by the viral protease; GAG-POL precursor is produced by ribosome frameshifting.

ENV - viral glycoproteins produced as a precursor (gp160) and processed to the external glycoprotein gp120 and the transmembrane glycoprotein gp41. The mature proteins are held together by noncovalent interactions; as a result, a substantial amount of gp120 is released in the medium. Gp120 contains the binding site for the CD4 receptor.

TAT - transactivator of HIV gene expression; one of the two necessary viral regulatory factors (TAT and REV) for HIV gene expression. Two forms are known, TAT-1exon (minor form) of 72 amino acids, and TAT-2exon (major form) of 86 amino acids. The electrophoretic mobility of these two forms in SDS gels is anomalous; they are approximately 16 kD and 14 kD in weight. Low levels of both proteins are found in persistently infected cells. TAT is localised primarily in the nucleolus/nucleus; it acts by binding to the TAR RNA element and activating transcription from the LTR promoter. Post-transcriptional effects of TAT have been postulated.

REV - the second necessary regulatory factor for HIV expression. A 19kD phosphoprotein localised primarily in the nucleolus/nucleus, REV acts by binding to RRE and promoting the nuclear export, stabilisation and utilisation of the viral mRNAs containing RRE.

VIF - viral infectivity factor, typically 23 kD; required for the efficient transmission of cell-free virus in tissue culture. In the absence of VIF, the produced viral particles are defective, while the cell-to-cell transmission of virus is not affected significantly. It has been reported that the cellular localisation is in the Golgi (VIF is not found in the virion).

VPR - virion-associated protein of unknown function found in HIV-1, HIV-2, SIVmac, and SIVmnd; typically 15kD. May be homologous to VPX. Also called 'RAP' for rapid.

VPU - "oUt" protein that promotes extracellular release of viral particles. Found only in HIV-1. Integral membrane phosphoprotein of 16kd; similar to M2 protein of influenza virus. It may be involved in ENV maturation. It is not found in the virion.

NEF - approximately 25 kD non-virion protein found in the cytoplasm of infected cells. Potentially myristoylated and associated with the inner plasma membrane. One of the first HIV proteins to be produced in the infected cells, it is the most immunogenic of the accessory proteins and may be used in the culture for diagnosis and staging of the disease. NEF is dispensable and probably suffers counter-selection during in vivo viral propagation. Recent evidence suggests that SIV NEF is required for viral propagation in vivo.

VPX - virion protein of 12 kD found only in HIV-2/ SIV and SIVAGM; not in HIV-1 or SIVmnd. Function unknown. VPU/VPX may be used to distinguish between HIV-1 and HIV-2 infection. (VPX may have some homology with VPR.)

TEV - (also known as tnv) a triparite 28 kD viral phosphoprotein produced very early in infection by some HIV-1 strains. Found primarily in the nucleolus/nucleus. TEV contains the first exon of TAT, a small part of ENV and the second exon of REV. It has both TAT and REV functions in the same polypeptide and can functionally replace both essential regulatory proteins of HIV-1.

STRUCTURAL PROTEINS - the products of GAG, POL and ENV genes, which are essential components of the retroviral particle.

REGULATORY PROTEINS - TAT and REV proteins of HIV/SIV and TAX and REX proteins of HTLVs; essential for viral expression in infected cells.

ACCESSORY PROTEINS - additional (non-regulatory) virion- and non-virion-associated proteins produced by HIV/SIV retroviruses: VIF, VPR, VPU, VPX, and NEF. Although the accessory proteins are not necessary for viral propagation in tissue culture, they have been conserved in the different isolates; this conservation and experimental observations suggest that their role in vivo is very important.

It will be appreciated that 'foreign' nucleic acids or proteins which bind to essential sites of the viral genome or to the viral DNA when synthesised will compete with the nucleic acids or proteins which bind to those sites and thus hinder replication. In general, such foreign entities will be specific to the viral material and will not interfere with the biosynthetic pathways of the host.

TAR is the binding site for TAT protein. A TAT protein analogue which binds to TAR but is otherwise inactive may be introduced by an appropriately modified gene. Similarly, a nucleic acid sequence which binds to TAR will prevent TAT binding.

Alternatively, sequences may be introduced which bind to important proteins such as TAT and REV, thereby "sequestering" them and preventing their function.

POL generates a number of viral enzymes, including reverse transcriptase and integrase. Nucleic acids which bind to those sections of POL which code for the

above enzymes will hinder both RNA transcription to DNA and integration to permit DNA translation.

Coding regions such as POL may also be disrupted by the introduction of genes encoding ribozymes capable of recognising and cleaving sequences in viral mRNA transcripts and/or in the viral genome thereby preventing synthesis of protein (eg. enzymes) crucial to viral function, as well as disrupting the integrity of unintegrated viral chromosomes.

As indicated above, DNA having any of the above binding capabilities or coding for RNA or proteins having such capabilities, when replicated together with the host genome, will serve to prevent viral replication. It may be preferable to use two or more such DNA sequences. They may be introduced into the host stem cells by any effective technique, most conveniently in capsid vectors.

However, production or expression of foreign nucleic acids or proteins in the host cell on a permanent basis may be harmful to cell function and there would be a considerable advantage if the exogenous genetic material could be switched on only when needed. One mechanism is to take advantage of the replication cycle of the genome of the capsid vector used to transduce the cells. When a pathogenic virus invades the host cell, certain proteins are produced at an early stage and are used to switch on later replication mechanisms. If the exogenous antiviral genetic material is under the control of a promoter sequence which is switched on by one of the switching proteins used by the invading virus, then as soon as the first invading virus produces the switching proteins, the antiviral blocking mechanism will be initiated to prevent any further virus replication.

In particular, it is advantageous to use a vector derived from a virus which is switched on by the same binding proteins as is the target virus. In some

instances, it is possible to use a modification of the genome of the target virus itself which is rendered unable to complete its full replication cycle but undergoes cycles of replication and possibly expression to produce the antiviral molecule. In the case of DNA viruses, the DNA control sequences including the sequence controlled by the viral switching protein are spliced to the antiviral gene in the correct reading frame and integrated into an appropriate vector.

In the case of HIV, the proteins TAT and REV function by binding to sites essential for viral replication as indicated above. If a modified form of a retrovirus such as HIV is used as the vector for introduction of the antiviral nucleic acid in such a way that the ds DNA integrated into the host genome contains genes corresponding to the TAT and REV binding sites TAR and RRE respectively, linked operatively in the pro-viral DNA to perform their native activating function, then invasion of the transformed cell by HIV producing the TAR and/or REV proteins at an early stage will switch on replication of the antiviral mRNA. Thus, if the vector is HIV modified by replacement of genes such as GAG, ENV, VIF and/or VPU (which are responsible for viral structural proteins) by the antiviral gene, the integrated dsDNA will be switched on when the invading HIV produces the TAR and/or REV proteins.

In the case of RNA viruses, the switching protein will bind only to RNA, whereas the antiviral gene and its control sequences need to be in the DNA of the genome of the host cell. In this case, it is necessary to modify a known control sequence compatible with the human genome to be responsive to the viral switching protein by DNA/protein binding.

The LTR (long terminal repeat) sequence of HIV includes the sequence U3-R-U5 and the binding site for tRNA primer essential for the first stage of replication is immediately 3' of this sequence. DNA binding to any

of the sequences will disrupt the HIV replication cycle by blocking extension of the tRNA primer as explained above.

The following DNA sequences may be transcribed to mRNA serving to block the HIV viral replication cycle:

Sequence 5'-3'	Complementary site[1]	Function 2
CAGTCAGTCAGTCAGTCAGT	-	Mismatched control
CTGGTCTAACCAGAGAGACC	1-20	Cap
TGAGGCTTATGCAGTGGGTT	54-73	5' untranslated
CTGCTAGAGATTTTCCACAC	162-181	Next to PBS
AAGTCCCTGTTCGGGCGCCA	182-201	PBS
GCGTACTGACCAGTCGCCGC	280-299	Splice donor
CTCGCACCCATCTCTCTCCT	327-346	gog initiator
TCTTCCCTAAAAAATTAGCC	1625-1644	Frame shift
TCTGCTGTCCCTGTAATAAA	4484-4503	Splice acceptor
GCCCCTTCACCTTTCCAGAG	4534-4553	Splice donor
CTGTTTTCCATAATCCCTAA	4613-4632	sor initiator
ATAGCAGAGTCTGAAAAACA	4961-4980	Splice acceptor
GAGATCCTACCTTGTTATGT	5035-5054	Splice donor
ACACCCAATTCTGAAAATGG	5349-5368	Splice acceptor
ACTGGCTCCATTTCTTGCTC	5403-5422	tat initiator
CCGCTTCTTCCTGCCATAGG	5548-5567	Splice acceptor/ or initiator
TACTACTTACTGCTTTGATA	5617-5636	Splice donor
TTCACTCTCATTGCCACTGT	5796-5813	emn initiator
GGAGGTGGGTCTGAAACGAT	7947-7966	Splice acceptor
TTGCCACCCATCTTATAGCA	8366-8385	3'-orf initiator
GGCAAGCTTTATTGAGGCTT	9183-9202	Poly(A) signal

1. Numbering of HIV RNA is that used in M.A. MUESENG et al., Nature 1985, 313, page 450-458;
2. Function of site to which the oligomer is complementary.

It may be desirable to combine two or more of such sequences to provide a more effective insert, for example, the sequences complementary to sites 162-181 and 182-201.

The vector used to transfect the stem cells may be one of those proposed for gene transfer into mammalian cells. Hantzopoulos et al. (Proc. Natl. Acad. Sci., 86, 3519-3523, 1989) have described a double copy retroviral vector wherein the transduced gene is inserted in the U3 region of the 3'-LTR. In the infected cell, the gene is duplicated and transferred to the 5'LTR where it is outside the retroviral transcriptional unit, thus avoiding any negative effects of that unit. Thus, the Mo-MuLV based vector (Coffin et a., RNA Tumour Viruses, Supplement, Cold Spring Harbour Lab) contains a unique XhoI site in a transcriptional orientation parallel to the viral transcriptional unit. The gene to be transferred is provided with XhoI sticky ends and inserted into the cleaved XhoI site.

Belmont et al. (Nature 322, 24 July 1986) have described a suitable retrovirus shuttle vector p.21P-SV(B). The SV(B) plasmid vector contains a XhoI restriction site which was cleaved, filled in with Klenow and treated with calf intestinal phosphatase (CIP). A plasmid containing the gene to be transduced is blunt end ligated into the cleaved vector. The plasmid so produced may then be introduced into a suitable capsid using a packaging cell line such as $\psi 2$ (Mann et al., Cell, 153-159) which may then be used to transduce human haematopoietic or other stem cells (Belmont et al., Nature, 24 July 1986).

As mentioned above, in the case of HIV, it is advantageous to use a HIV-based vector. In addition to previously-mentioned advantages, HIV-based vectors can integrate in non-dividing cells, minimising the handling of stem cells. Several HIV-based vectors have been published (Buchschacker and Panganiban, 1992 J. Virol,

66: 2731-9).

More than one gene may be inserted in the vector for replication and/or expression. The insert may thus include two or more antiviral genes which may be under control of separate promoters to ensure separate replication or expression. Similarly, multiple copies of the same gene may be introduced to enhance the antiviral effect.

A useful back-up strategy may be to include in the vector a mechanism which will be activated to kill infected cells if viral growth is not sufficiently checked. Thus for example a toxin-encoding sequence, eg. Diphteria toxin may be incorporated in such a manner that it is transcribed only if the virus continues to grow within the infected cell, for example by making use of a switching system such as previously described.

Transfection of the stem cells may be by any conventional method known in the art. This may include for example repeated infection of bone marrow cells in culture with packaged construct (Bordignon et al 1989, P.N.A.S., USA 86: 6748-52) or coculture with a packaging cell line (Bodine et al., 1990, PNAS USA 87: 3738-42). Alternatively, the construct may be introduced by gene cannon (Klein et al., 1987, Nature 327: 70-3). To enhance retroviral gene transfer, the stem cell population can be prestimulated with different growth factor combinations like CSF/IL-6 or IL-3/IL-3 (Luskey et al., Blood 1992, 392-402).

The following Examples are given by way of illustration only:

Example 1

The oligonucleotide shown below and its complementary strand are synthesised using an Applied Biosystems 381A DNA synthesiser under the conditions prescribed by the manufacturer.

CTGCTAGAGATTTTCCACACAAGTCCCTGTTCGGGGCGCCCA

The double stranded antiviral DNA (AV) insert is blunt end ligated to the simian virus 40 (SV40) promoter by the method of Belmont et al. (Nature, 322, 24 July 1986) to produce an insert SV40AV.

Retroviral vector pZipDHFR* (Williams et al., Proc Natl. Acad Sci., USA, 1986, 2566-2570) is cleaved with ZhoI and the SV40AV insert is introduced to generate pZipDHFR*SV40AV.

The recombinant plasmid is transfected into packaging cell line ψ2 (Mann et al., Cell 33, 153-159) to generate a helper free viral stock. Transfection is achieved by calcium phosphate precipitation and transformed clones are selected in Dulbecco's modified Eagle medium containing 10% dialysed calf serum and 0.25 μM methotrexate. Clones producing up to 5 x 10^5 colony forming units (cfu) per ml are obtained in this way.

Haematopoietic stem cells produced in accordance with Example 1 of WO91/09938 of Dynal AS are cultivated according to the method of Andrews and then co-cultivated with a monolayer of 10^6 cells of the packaging cell line ψ2 (transfected with the plasmid) for 24 hours in the presence of Polybrene (2 μg/ml). After transduction, the stem cells are removed from the monolayer, grown in culture for 24 hours to allow adherence of any fibroblasts, pelleted and resuspended in fresh medium.

Example 2

Principle

Haematopoeitic stem cells are obtained in accordance with Example 1 of WO91/09938 of Dynal AS. Into these cells is introduced a construct consisting of a retroviral vector and three transcribed regions under control of the HIV TAT/TAR system. One of the transcribed regions encodes an RRE decoy, a short sequence expressed at high level and containing RRE, thus preventing Rev mediated transport of unspliced or partially spliced mRNA into the cytoplasm by sequesering Rev (Schwartz et al 1992, J-Virol 66: 150-9). The second of the transcribed regions encodes a ribozyme recognizing and cleaving a sequence in the HIV pol gene mRNA (Wong-Staal 1992, Lecture at the 1992 Cold Spring Harbour Laboratory's meeting on gene therapy, sited in Science 258: 745). This will inhibit synthesis of enzymes crucial to HIV function, as well as disrupt the integrity of unintegrated viral chromosomes. The third of the transcribed regions encodes Diphteria toxin A-chain, engineered in such a way as to kill off the cell if infection proceeds in spite of the ribozyme and the RRE decoy.

Vector

The HIV based vector GB102 (Buchschacher & Panganiban 1992, J-Virol 66: 2731-9), modified to hold four transcribed regions, each independently under control of the TAT/TAR system, is used. This is done by the introduction of a polylinker in the BssHII site of GB102.

RRE decoy

The RRE decoy is generated by placing RRE, correctly orientated, in the polylinker region downstream of the HIV LTR and TAR regions of GB102. The

other gene constructs are placed downstream of RRE, away from the LTR/TAR combination of the vector.

Ribozyme

It has been shown that a suitable ribozyme can reduce HIV protein production by 95% (Wong-Staal 1992, Lecture at the 1992 Cold Spring Harbour Laboratory's meeting on gene therapy, sited in Science 258: 745). Assembly of fresh virus particles should also be inhibited through endonucleolytic cleavage of the HIV RNA chromosome. The ribozyme construct is transcribed from chicken beta-actin promoter, which can be activated by TAT binding to TAR (Han et al 1991, Nucleic-Acids-Res 19: 7225-9), and TAR is included in the construct. The promoter-coding region assembly is inserted into the polylinker.

Diphtheria toxin

The rationale behind including the diphteria toxin encoding sequence is that it should be transcribed as long as there is TAT present in the cell. It is dependent on Rev function for its export from the nucleus, and thus is expressed as a protein only after the RRE decoy system has broken down, either by a mutation in the decoy or its TAR region or by a double mutation in TAT and the viral TAR region. This is achieved by placing the A chain part of the diphteria toxin cDNA sequence (Bishai et al 1987, J. Bacteriol 169: 1554-63) downstream of TAR, RRE and INS-1 under the control of chicken beta-actin promoter, which can be activated by TAT binding to TAR (Han et al 1991, Nucleic-Acids-Res 19: 7225-9). INS-1 is a region that confers a high degree of instability to the RNA molecule of which it is a part, unless it is bound to Rev (Schwartz et al 1992, J-Virol 66: 150-9). The promoter-TAR-RRE-INS-1-coding region is inserted into the polylinker.

Selectable marker

In order to select transfected cells in culture, the construct also contains the selectable marker bacterial xanthineguanine phosphoribosyltransferase (XGPRT). The introduction of this enzyme makes the cells able to grow on a medium containing adenine, xanthine, mycophenolic acid and aminopterin (Mulligan & Berg 1981, Proc-nat-Acad-Sci-USA 78: 2072-6). If desirable, the promoter controlling the XGPRT expression can be inducible. The promoter and XGPRT coding region is inserted into the polylinker.

Transfection

The construct is introduced by the gene cannon technique (Klein et al 1987, Nature 327: 70-3). To enhance retroviral gene transfer, the stem cell population is prestimulated with growth factor combinations (CSF.IL-6 or IL-3/IL-3) (Luskey et al., Blood 1992, 392-402).

Claims

1. A mammalian stem cell transduced with one or more replicable vectors carrying antiviral DNA which by production of RNA or DNA or by expression of one or more proteins, will block replication of at least one virus infecting mammalian cells deriving from said stem cells.

2. A mammalian stem cell as claimed in claim 1 which is a haematopoietic stem cell.

3. A mammalian stem cell as claimed in claim 2 in which said virus is HIV or HTLV.

4. A mammalian stem cell as claimed in claim 1 which is an endothelial stem cell.

5. A mammalian stem cell as claimed in claim 4 in which said virus is a hepatitis virus.

6. A mammalian haemopoietic stem cell as claimed in claim 2 which carries the CD34 antigen but not antigens DP and DQ of the MHC Class II group.

7. A mammalian stem cell as claimed in any of the preceding claims transduced with a vector providing a gene expressing a modified form of reverse transcriptase which is inactive as an enzyme but retains binding specificity for the reverse transcriptase binding site.

8. A mammalian stem cell as claimed in any of the preceding claims transduced with a vector providing a gene transcribing the t-RNA initiating transcription of the target virus modified by blocking the 3'-hydroxy terminal or otherwise blocking 3'-chain extension.

9. A mammalian stem cell as claimed in any of the preceding claims transduced with a vector providing a gene transcribing an RNA sequence which blocks the TCAG integration sites on the host genome and thus blocks viral integration.

10. A mammalian stem cell as claimed in any of the preceding claims transduced with a vector providing a gene expressing a TAT protein analogue which binds to the TAR region of the retroviral genome but lacks TAT activity.

11. A mammalian stem cell as claimed in any of the preceding claims transduced with a vector providing a gene comprising one or more of the following DNA sequences

CAGTCAGTCAGTCAGTCAGT
CTGGTCTAACCAGAGAGACC
TGAGGCTTATGCAGTGGGTT
CTGCTAGAGATTTTCCACAC
AAGTCCCTGTTCGGGCGCCA
GCGTACTGACCAGTCGCCGC
CTCGCACCCATCTCTCTCCT
TCTTCCCTAAAAAATTAGCC
TCTGCTGTCCCTGTAATAAA
GCCCCTTCACCTTTCCAGAG
CTGTTTTCCATAATCCCTAA
ATAGCAGAGTCTGAAAAACA
GAGATCCTACCTTGTTATGT
ACACCCAATTCTGAAAATGG
ACTGGCTCCATTTCTTGCTC
CCGCTTCTTCCTGCCATAGG
TACTACTTACTGCTTTGATA
TTCACTCTCATTGCCACTGT
GGAGGTGGGTCTGAAACGAT
TTGCCACCCATCTTATAGCA
GGCAAGCTTTATTGAGGCTT

12. A mammalian stem cell as claimed in any of the preceding claims transduced with a vector providing a gene comprising the DNA sequence

CTGCTAGAGATTTTCCACACAAGTCCCTGTTCGGGGCGCCCA

13. A mammalian cell as claimed in any of the preceding claims wherein said gene is operatively linked in frame to a promoter capable of promoting transcription in said stem cell.

14. A mammalian cell as claimed in any of the preceding claims transduced by a viral capsid vector carrying said gene.

15. A mammalian stem cell as claimed in claim 1 transduced with a vector whereby the antiviral DNA includes one or more promoter or other sequences activated by proteins produced by an invading virus so that antiviral RNA, DNA or protein is produced only when so activated.

16. A mammalian stem cell as claimed in claim 15 transduced with a retroviral vector comprising the TAR and/or RRE sequences operatively linked to effect activation of mRNA synthesis when binding to the TAT and/or REV proteins produced by an invading retrovirus, the vector containing an antiviral RNA sequence in place of one or more RNA sequences of the native retrovirus so that mRNA is produced which prevents replication of the invading virus.

17. A mammalian stem cell as claimed in claim 16 in which the retroviral vector is derived from HIV.

18. A mammalian stem cell as claimed in any preceding claim wherein the vector further comprises a sequence which is activated to kill virus-infected cells if viral

replication is not blocked.

19. A method of combating viral infections in mammals in which a population of stem cells as claimed in claim 1 is introduced into said mammal whereby said cells proliferate and differentiate into progeny cells protected against said target virus.

20. A method as claimed in claim 19 in which the mammal is a human, the cells are haemopoietic stem cells and said target virus is HIV.

21. A method of producing a stem cell as claimed in claim 1 wherein a selected population of mammalian stem cells is transduced with one or more replicable vectors carrying antiviral DNA which by production of RNA or DNA or by expression of one or more proteins, will block replication of at least one virus infecting mammalian cells deriving from said stem cells.

1/1

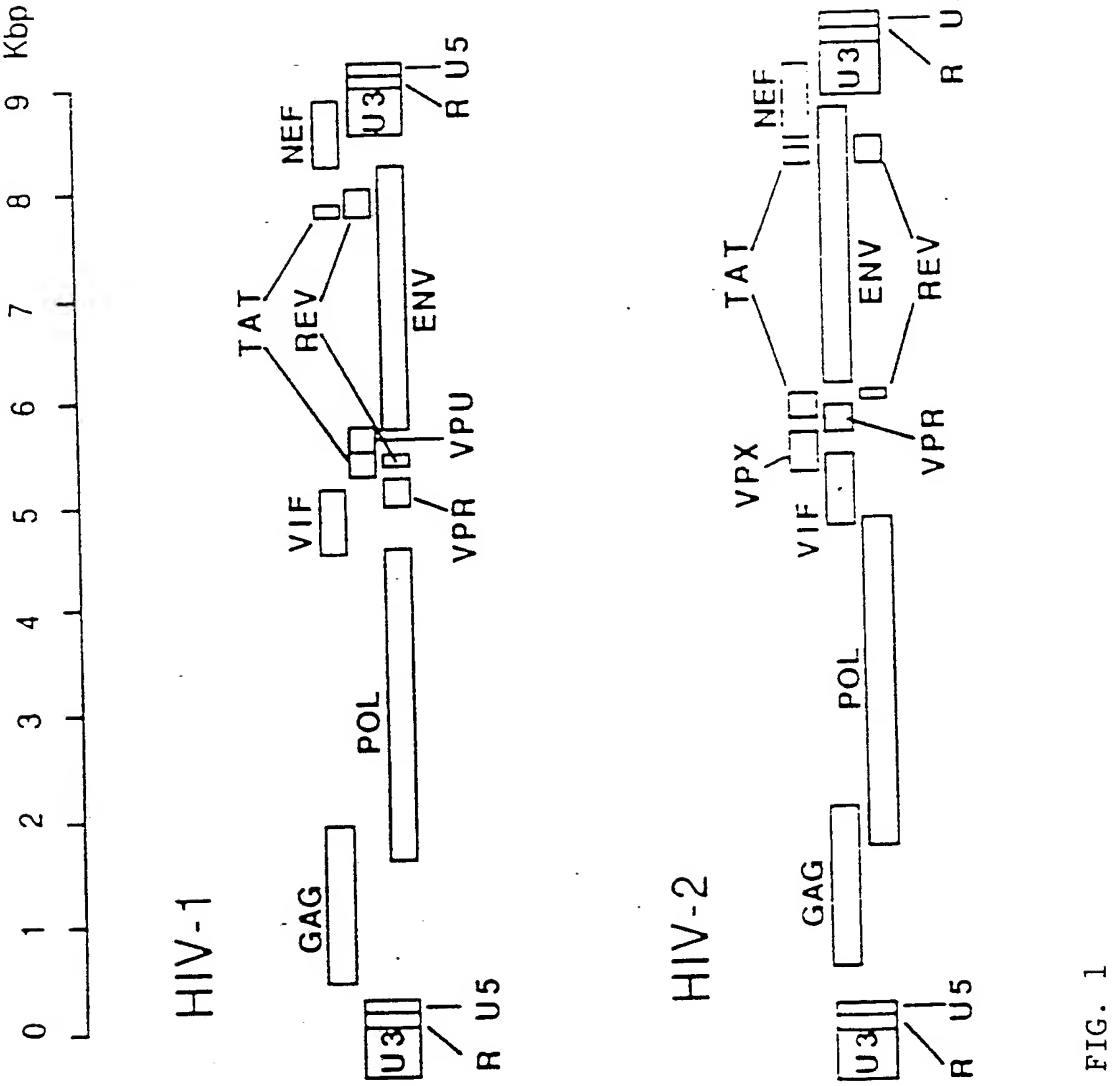
0 1 2 3 4 5 6 7 8 9 Kbp
HIV-1
GAG
POL
VIF
VPR
TAT
REV
VPU
ENV
NEF
U3
R U5
HIV-2
GAG
POL
VIF
VPX
VPR
TAT
REV
ENV
NEF
U3
R U5

FIG. 1

INTERNATIONAL SEARCH REPORT

International Application No PCT/EP 92/02787

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all)[6]

According to International Patent Classification (IPC) or to both National Classification and IPC

Int.Cl. 5 C12N15/11; C12N15/48; C12N15/12; C12N15/31
C12N15/86; C12N9/00; C12N5/10; A61K48/00

II. FIELDS SEARCHED

Minimum Documentation Searched[7]

Classification System	Classification Symbols
Int.Cl. 5	A61K ; C12N ; C07K

Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched[8]

III. DOCUMENTS CONSIDERED TO BE RELEVANT[9]

Category °	Citation of Document,[11] with indication, where appropriate, of the relevant passages[12]	Relevant to Claim No.[13]
X	WO,A,9 110 453 (THE UNITED STATES OF AMERICA, DEPARTMENT OF COMMERCE) 25 July 1991 see the whole document	1-3, 13-17, 19-21
X	WO,A,8 904 168 (BIOCYTE CORPORATION) 18 May 1989 see claims 10,12,13,26,34,49,55-59	1-3, 19-21
Y	see the whole document	1-3,13, 17,19-21
Y	EP,A,0 300 687 (CITY OF HOPE) 25 January 1989 see the whole document	1-3,13, 17,19-21

-/--

° Special categories of cited documents :[10]

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report
02 APRIL 1993	16. 04. 93
International Searching Authority	**Signature of Authorized Officer**
EUROPEAN PATENT OFFICE	CHAMBONNET F.J.

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)

Category °	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No.
X	WO,A,8 912 461 (ST. LOUIS UNIVERSITY) 28 December 1989 see claims	1-3,11, 13,19-21
Y	see the whole document	1-3,13, 17,19-21
E	DE,A,4 126 484 (BAYER AG.) 11 February 1993 see the whole document	1-3,8, 11-14, 19-21
A	WO,A,9 109 938 (DYNAL A.S.) 11 July 1991 cited in the application see abstract	6
X	PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES OF USA. vol. 88, no. 10, May 1991, WASHINGTON US pages 4313 - 4317 HAN, L. ET AL. 'Inhibition of Moloney murine Leukemia virus-induced leukemia in transgenic mice expressing antisense RNA complementary to the retroviral packaging sequences' see the whole document	1

INTERNATIONAL SEARCH REPORT

:national application No.

PCT/EP 92/02787

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. [X] Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:
Remark: Although claims 19 and 20 are directed to a method of treatment of the human/animal body the search has been carried out and based on the alleged effects of the compound/composition.

2. [ ] Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. [ ] Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. [ ] As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. [ ] As all searchable claims could be searches without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. [ ] As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. [ ] No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

[ ] The additional search fees were accompanied by the applicant's protest.

[ ] No protest accompanied the payment of additional search fees.

ANNEX TO THE INTERNATIONAL SEARCH REPORT ON INTERNATIONAL PATENT APPLICATION NO.

EP 9202787
SA 68406

This annex lists the patent family members relating to the patent documents cited in the above-mentioned international search report.
The members are as contained in the European Patent Office EDP file on 02/04/93
The European Patent Office is in no way liable for these particulars which are merely given for the purpose of information.

Patent document cited in search report	Publication date	Patent family member(s)		Publication date
WO-A-9110453	25-07-91	AU-A-	7074091	05-08-91
		EP-A-	0511285	04-11-92
WO-A-8904168	18-05-89	US-A-	5004681	02-04-91
		AU-A-	2610288	01-06-89
		EP-A-	0343217	29-11-89
		JP-T-	3501207	22-03-91
		US-A-	5192553	09-03-93
EP-A-0300687	25-01-89	US-A-	5110802	05-05-92
WO-A-8912461	28-12-89	AU-A-	3756789	12-01-90
DE-A-4126484	11-02-93	None		
WO-A-9109938	11-07-91	AU-A-	7069791	24-07-91
		EP-A-	0507839	14-10-92